The potassium channel opener levcromakalim causes expansive remodelling of experimental vein grafts

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Background: **Maintenance of luminal area is essential for the optimal performance of venous bypass grafts. However, injury and response to the arterial circulation evoke vascular remodelling that favors intimal hyperplasia, with luminal encroachment and inward remodelling. Potassium channel-opening drugs reduce tissue workload and peripheral vascular resistance and through these mechanisms could favor outward or expansive remodelling of vein grafts. We tested the hypothesis that levcromakalim, a potassium channel opener, would enhance expansive remodelling in vein grafts.**

Methods: **A randomized, double-blind, placebo-controlled trial was conducted in 33 rats with vena cava-to-aorta bypass grafts. Drugs were administered via osmotic pump for 7 days after surgery. Half the cohort had bromodeoxyuridine (BrdU) infused at day 6. Morphometric analysis was conducted of pressure perfusion-fixed grafts harvested at 1 week and 4 weeks.**

Results: **At 1 week, lumen area was similar in both groups (1.82 ± 0.39 mm^2 placebo vs 1.85 ± 0.36 mm^2 levcromakalim), although medial cell density and BrdU staining were significantly increased in the placebo group. At 4 weeks, lumen area was unchanged in the placebo group (1.88 ± 0.51 mm^2) but had increased to 2.32 ± 0.46 mm^2 in the levcromakalim group ($P = .039$ vs 1 week), with a very significant reduction in the intimal area (levcromakalim, 0.06 ± 0.02 mm^2 vs placebo, 0.33 ± 0.17 mm^2; $P = .001$).**

Conclusions: **Early, short-term treatment with levcromakalim favors expansive remodelling of experimental vein grafts to mimic the effect of external stenting. This expansive remodelling was associated with a reduction in medial cell proliferation at 1 week.**

Clinical Relevance: **Critical limb ischemia can be treated by bypass surgery or angioplasty, but inward remodelling with restenosis is a common problem. There has been little previous experimental work to identify treatments associated with expansive remodelling, which would increase the chances of vessel patency. Here, in a randomized trial, we show that short-term treatment with a potassium channel opener (a class of drug that can be used to treat hypertension) results in strong, expansive remodelling, with increases the lumen area and graft size of experimental vein grafts by >25%. (J Vasc Surg 2006;44:159-65.)**

The concept of using saphenous vein for bypassing diseased coronary or peripheral arteries was a major advance for cardiovascular surgery. That vein, with anatomic and physiologic functions suited to a low-pressure minimally pulsatile circulation, can be excised and used as an arterial conduit is remarkable, this being achieved through a process of vascular remodelling.[1] Saphenous vein has myogenic tone and constricts in response to acute increases in intraluminal pressure.[2] To prevent this occurring in clinical practice, vein is stretched beyond its elastic limit or is treated with papaverine before implantation. Overdistension may leave a vessel of larger diameter than the arteries to which it is anastomosed.

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Unsurprisingly, intimal hyperplasia results in the inward remodelling of saphenous vein grafts. Excessive intimal hyperplasia narrows the lumen of venous bypass grafts so that symptoms recur and graft failure is a likely sequela. Graft failure occurs in up to 30% of venous bypass grafts, intimal hyperplasia being a common cause of the graft failures occurring between 1 and 24 months after surgery.[3,4]

A large body of work of experimental work has been directed at attenuating the development of intimal hyperplasia in vein grafts, with intimal thickness being the principal outcome measure of most studies.[5-7] Surprisingly lumen area, a critical determinant of graft function, has received far less attention. External stenting of vein grafts is one of the few treatments that has been reported to lead to expansive remodelling, or an increase in the lumen area.[8,9]

External stenting would appear to be an important advance to improve the outcome of venous bypass grafts in patients. Unfortunately, technical difficulties in the diseased human vasculature, together with an increasing risk of infection of tissue already ischemic, might underlie the absence of successful clinical trials of external vein graft stenting. We therefore searched for a possible pharmaceutic agent that might, like external stenting, cause expansive

remodelling of vein grafts. Because the early hemodynamic and metabolic changes in vein grafts trigger the long-term vascular remodelling responses,[10-13] such an agent need only be administered for a short time.

Adenosine triphosphate (ATP)-dependent potassium (K_{ATP}) channels link cellular metabolism to membrane function and cellular excitability. Potassium channel-opening drugs such as pinacidil and diazoxide are used clinically, usually for treatment of hypertension. They reduce workload in angina, hypertension, and peripheral vascular disease, and they are vasodilators and reduce peripheral resistance.

K_{ATP} channels are present in human saphenous vein, and levcromakalim (a benzopyran derivative) is the most potent of the potassium channel openers for this tissue.[14] The metabolic effects to reduce oxidative stress and preserve mitochondrial function of these benzopyran derivatives suggest that they may be useful in ischemic preconditioning. K_{ATP} channels are also present in endothelium, where they are upregulated by shear stress, and in vascular smooth muscle.[15,16]

For all of these reasons, we considered that potassium channel openers could provide a metabolic mimic of external stenting. Here, we test the hypothesis that levcromakalim causes expansive remodelling of experimental vein grafts.

METHODS

Animals and drug delivery. Studies were performed using syngenic male Lewis rats weighing 200 to 250 grams. All animals received humane care in accordance with the Home Office Animals (Scientific Procedures) Act 1986 (HMSO 1990). Experiments were conducted under Project Licence PPL 70/4370, Personal Licence PIL 70/15215. Animals were randomized by an independent scientist to receive levcromakalim or a placebo. Osmotic pumps (ALZET model 2ML1, Alza Corporation, Mountain View, Calif) containing either levcromakalim (1.62 mg/mL, 20% v/v ethanol) or an identical-looking placebo (20% v/v ethanol) were implanted subcutaneously 24 hours before aortocaval grafting. A tunnelled catheter enabled intravenous drug or placebo delivery via the external jugular vein (n = 33).

Levcromakalim assay. Plasma levcromakalim was assayed using high performance liquid chromatography (HPLC)-multiple reaction monitoring in the presence of another benzopyran, HOE234, a gift from Hoechst Pharmaceuticals (Frankfurt, Germany) as an internal standard. Briefly, plasma (75 μL) was treated with ice-cold acetonitrile (0.4 mL) to precipitate proteins, extracted with Oasis cartridges (Waters, St Quentin-en-Yvelines, France) and purified by HPLC on a Hichrom rpb column (10 cm × 2 mm) eluting at 0.1 mL/min with a linear gradient of acetonitrile:water:formic acid (30:70:0.1-60:40:0.1). Levcromakalim eluted at 11.5 minutes and HOE234 at 16.2 minutes. The eluate was passed into the +ve ion electrospray source of a Micromass Quattro II triple quadrupole mass spectrometer (Waters, St Quentin-en-Yvelines, France) for analysis in the multiple reaction-monitoring mode using ion transitions m/z 287→160 (levcromakalim) and m/z 402-275 (HOE234). Levcromakalim was quantified against a 6-point extracted standard curve. The limit of detection was set at 2.5 ng/75 μL and the limit of quantification at 5 ng/75 μL with a corresponding coefficient of variation of 10.4%.

Operative procedures. Aortocaval bypass grafts were performed by harvesting the supradiaphragmatic portion of the inferior vena cava (IVC) from a donor rat and inserting a 1- to 1.2-cm segment length (diameter up to 20% greater than the recipient aorta) into the infra-abdominal aorta of the recipient rat under ×10 magnification using end-to-end anastomoses with interrupted 10-0 nylon sutures.[17] Osmotic pumps were removed at 7 days. Plasma samples were taken on day 1 and day 7 for levcromakalim assay. Rats, for sacrifice at 7 days, received intraperitoneal injections of bromodeoxyuridine (BrdU) 100 mg/kg at 17 hours, 9 hours, and 1 hour before graft harvesting. Grafts were harvested at 1 and 4 weeks by using perfusion fixation with 10% formaldehyde at 90 mm Hg.

Sectioning and staining. Grafts were divided into proximal, mid, and distal segments after excision of anastomoses and were embedded in paraffin. Consecutive mid-graft 3 μm sections (n = 36) were stained with hematoxylin and eosin (H&E) and elastic van Gieson (EVG) at six-section intervals. Immunostaining for BrdU was performed on similar mid-graft sections (n=6 for each graft) from 1-week grafts.[18]

Image analysis. Microscopic images of the vein grafts were analyzed by a computer-assisted image analysis system (AnalySIS 3.1, Soft Imaging System, Analysis Imaging, Munster, Germany). All measurements were made with the observer blind to the treatment group. Captured images of EVG sections at ×4 magnification were used for morphometric analysis (n = 6 per graft). The EVG stained sections were used to define the area enclosed by the endothelium as the lumen, the area between the endothelium and the internal elastic lamina defined the intima, the area between the internal and external elastic lamina (EEL) defined the media, and the overall vein graft size was measured as the area contained within the EEL (Fig 1). The elastic lamina boundaries were selected by locating the innermost fenestrated elastic lamina adjacent to the endothelium or intima (IEL) and the outermost lamella defining the border between muscular media and the looser connective tissue of the adventitia (EEL).

In the 4-week group, morphometric parameters were assessed in proximal, mid, and distal graft segments, and intimal/medial area ratios were calculated. In the 1-week group, mid-graft sections were analyzed for luminal area and combined intimal and medial area, as a discrete intimal layer could not be identified.

Captured images of H&E stained sections at ×40 magnification were used for quantification of cell density. Six fields in six consecutive sections were analyzed per mid-graft cross section at 45° intervals (n = 36 per graft segment). Intimal and medial boundaries were confirmed

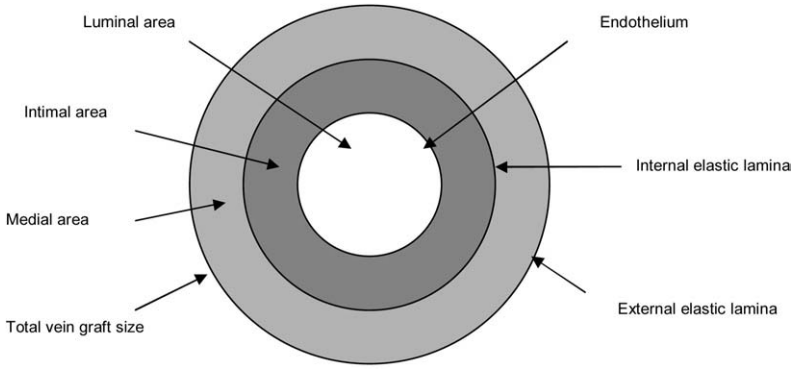

Fig 1. Schematic representation of anatomic landmarks and measurements made.

by comparison with sequential sections stained for elastin. After defining intimal and medial regions, nuclear counting was performed, and cell density measurements were calculated. BrdU staining was quantified as a marker of medial cell proliferation in 1-week grafts and quantified on a scale of 0 (none) to 3 (most) in the medial layer of the mid-graft wall.[18]

Statistics. Power calculations were based on a 25% increase in luminal area, giving a sample size of eight a power of 80% at the 5% significance level. Means and standard deviations were calculated for measured parameters. Comparisons between groups for morphometric and cell density analyses were made using the two-sample Wilcoxon rank-sum (Mann-Whitney) test. BrdU staining scores between groups were compared using Fischer's exact test.

RESULTS

Thirty-three animals successfully recovered from anesthesia after aortocaval bypass. Operative mortality and long-term survival were very similar in the two groups. Preoperatively, the placebo and levcromakalim groups weighed 232 ± 6.3 grams and 230 ± 10.3 grams, respectively, and there was no difference between the groups in weight gain at either 1 week or 4 weeks after surgery. One animal from the levcromakalim group developed sepsis secondary to pump infection at day 21, and another animal from the placebo group had an occluded graft at 4 weeks; both were excluded from the morphometric analysis.

The infusion dose of levcromakalim (860 ng/min/kg to achieve a steady state plasma concentration of 32.7 ng/mL) was selected as a result of pharmacokinetic and pharmacodynamic studies that showed levcromakalim has a half-life of 32.5 minutes and a limited hypotensive effect in the rat at a plasma concentration of 33 ng/mL (Fig 2). In animals receiving levcromakalim, the target plasma concentration of plasma levcromakalim was 33ng/mL, a concentration at which K_{ATP} channels appeared to be activated (relaxation of vein rings precontracted with phenylephrine) but with only a minimal reduction (<5%) in mean arterial blood pressure.

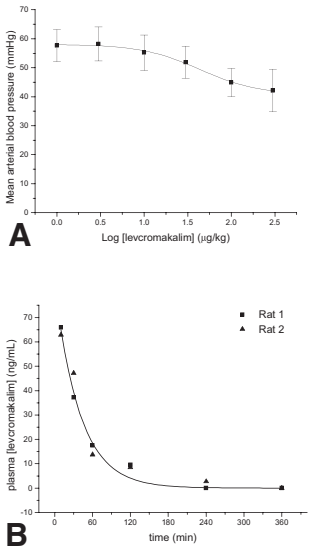

Fig 2. Pharmokinetic studies of levcromakalim. **A** Hypotensive effect of levcromakalim in anesthetized normotensive rats. Blood pressure (mean ± SD) was transduced from an intra-aortic line, in 3 rats, in response to intravenous levcromakalim. **B** Rat plasma levcromakalim concentration following a single bolus of levcromakalim (100 μg/kg). The decay curve was identical in 2 rats, giving a half-life of 32.5 minutes.

EVG staining of vein ring sections showed the presence of elastic laminae (Fig 3). The mean levcromakalim concentration at day 1 was 36.8 ± 10.7 ng/mL, and it was 33.7 ± 11.7 ng/mL at day 7. No levcromakalim was detected in plasma samples taken from the control group at either day 1 or day 7. An initial assessment of intimal and medial areas at proximal, mid-graft, and distal graft segments in 4-week vein grafts found no significant differences in these areas for the different graft segments; therefore, the mid-graft section was chosen for all further detailed analyses.

The medial thickness of normal IVC at grafting was 40 μm, but by 1 week, the medial thickness was much greater than 40 μm in both levcromakalim and placebo groups (Fig 4). Although the IEL could be defined easily at this time

Fig 3. Rat thoracic vena cava. Elastic van Gieson staining shows the elastic laminae. The scale bar represents 50 μ.

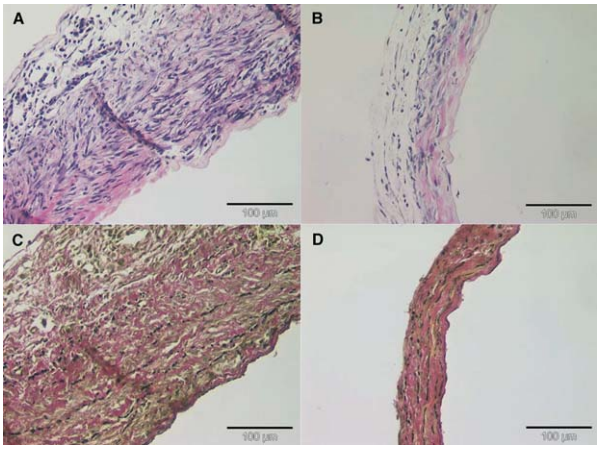

Fig 4. Vein grafts at 1 week. Examples from the placebo group are shown in **A** stained with hematoxylin and eosin (H&E) and in **C** stained with elastic van Gieson (EVG). Examples from the levcromakalim group are shown in **B** (H&E) and **D** (EVG). Note the periadventitial stripping (the result of processing for histology) in **D**. The scale bar represents 100 μ.

point, the neointimal layer was barely discernible. Therefore, combined intimal plus medial areas were calculated by subtracting the luminal area from the area contained within the EEL. Although the adventitia was intact at vein graft harvest, the adventitial layer was stripped off during processing in some histologic sections (Fig 4, *D*). Therefore, morphometric analysis of the adventitia was not included. All the morphometric results at 1 week are given in Table I.

The reduction in medial cell density in the levcromakalim group was confirmed by BrdU staining, which was observed in the media and adventitia of 1-week grafts. Medial BrdU staining is presented as a score because of weak H&E counterstaining in some sections. The median proliferation scores for the medial layer were +1 in the levcromakalim group and +3 in the placebo groups (P = .02), and representative staining is shown in Fig 5.

By 4 weeks, a separate intimal area was clearly discernible in all vein grafts, although the intimal thickening appeared much greater in the placebo group than in the levcromakalim group (Fig 6). The increased lumen area of the levcromakalim group is evident in Fig 6, which summarizes the morphometric findings. Detailed morphometric results at 4 weeks, including intimal area, are given in Table II.

Remodelling characteristics were assessed by comparing changes in lumen area and total vein graft size between 1 and 4 weeks (Fig 7). Vein graft size and lumen area significantly increased in the levcromakalim treated group during this time. Consistent with an expansive remodelling process, the mean increase in vein graft size was 0.65 mm^2 (28%) (P = .002), and the mean increase in lumen area was 0.46 mm^2 (26%) (P = .039). No significant change was noted in lumen area or vein graft size in the placebo group between 1 and 4 weeks.

DISCUSSION

The hypothesis that the K_{ATP} channel opener levcromakalim favors the expansive remodelling of vein grafts is supported by the results of this study, which show that a modest dose of levcromakalim was associated with a >25% increase in both vein graft and lumen size at 4 weeks, these changes being accompanied by a 5-fold reduction of intimal area. Although expansive remodelling is a desirable clinical outcome, most previous experimental studies have focused on the development of intimal hyperplasia rather than vein graft dimensions.[4]

It is encouraging that lumen and vein graft area are becoming recognized as important outcome measures. For instance, one of the early events underlying the development of intimal hyperplasia is likely to be the formation of microthrombi on the damaged endothelial surface.[19] A recent study has indicated that thrombin inhibitors cause expansive remodelling of expansive vein grafts.[20] Other early events in vein graft remodelling include altered cell-signalling processes leading to the degradation of muscle actin filaments in response to mechanical stretch and enhanced smooth muscle cell proliferation.[21]

The synergistic effects of levcromakalim to cause vasodilation and excitation-metabolic coupling are likely to minimize workload on the new vein graft and hence control cell-signalling events to limit degradation of actin filaments. This in turn appears to have led to a reduced medial cell proliferation and BrdU staining. Levcromakalim also decreases peripheral vascular resistance to encourage high flow through the graft, which may contribute to favorable expansive remodelling.[22]

Intimal hyperplasia formation is associated with inward, constrictive remodelling processes, with progressive luminal compromise leading to the development of hemodynamically significant vein graft stenoses strongly impli-

Table I. Morphometric measurements and cell count in vein grafts at 1 week

	Placebo (n = 8)	*Levcromakalim (n = 8)*	P
Combined intimal and medial area (mm^2)	1.07 ± 0.28	0.38 ± 0.05	.001*
Lumen area (mm^2)	1.82 ± 0.39	1.85 ± 0.36	.898*
Vein graft size (mm^2)	2.81 ± 0.59	2.23 ± 0.36	.029*
Medial cell density (cells/mm^2)	5530 ± 760	3420 ± 280	.001*
BrdU score (median)	+3	+1	.02†

BrdU, Bromodeoxyuridine.
*Mann-Whitney.
†Fischer's exact.

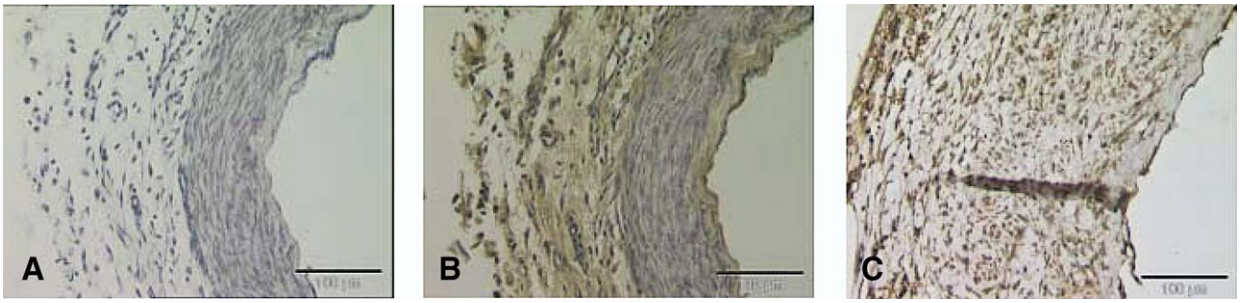

Fig 5. Bromodeoxyuridine (BrdU) staining of vein grafts at 1 week. **A,** Control 0 staining. **B,** Example of levcromakalim treated group showing +1 medial staining for BrdU. **C,** Example from placebo group showing +3 medial staining for BrdU. The scale bar represents 100 μm.

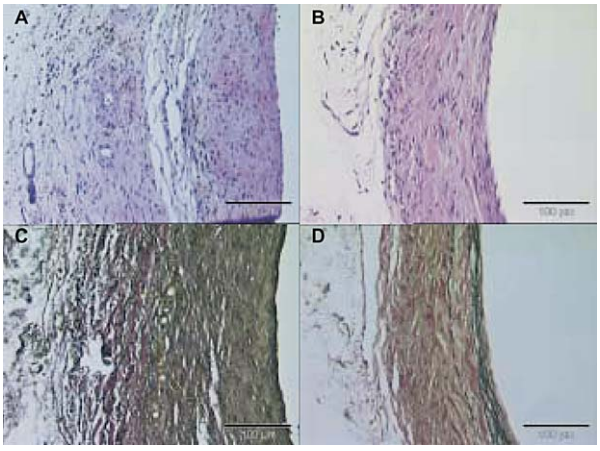

Fig 6. Vein grafts at 4 weeks. Examples from the placebo group are shown in **A** stained with hematoxylin and eosin (H&E) and in **C** stained with elastic van Gieson (EVG). Examples from the levcromakalim group are shown in **B** (H&E) and **D** (EVG). The scale bar represents 100 μm.

cated in the etiology of vein graft failure. Although geometric remodeling processes in experimental vein grafts have rarely been measured, the features that characterize expansive arterial remodelling have been recently described.

There is clinical evidence that expansive remodelling of vein grafts occurs even in diseased vein grafts.[23] The balance of proteolytic and inflammatory activities is one important item of discussion,[24-26] although blood flow also is likely to have an important influence on remodelling. Reduced late constrictive remodelling has been reported in experimental arterial angioplasty after early metalloproteinase inhibition.[27]

Although the conclusions of our study are supported by strong methodology, particularly the double-blinding aspects, whereby an independent person randomized the animals to treatment with placebo or levcromakalim and the observers remained ignorant of treatment allocation until the statistical analysis of morphometric results had been completed, the study was conducted in healthy, nonatherosclerotic vessels. Our choice of the 4-week time point as one where the remodelling process has stabilized is supported by the evidence of others.[28] However, most previous murine studies have used vena cava-to-carotid bypass grafts. Our choice of vena cava-to-aortic grafts permitted greater flexibility of anastomotic techniques, permitting end-to-end anastomoses in a high flow situation, and perhaps this contributed to similar levels of intimal hyperplasia identified along the graft in contrast to the vena cava to carotid model where intimal hyperplasia is greatest at the anastomoses.[29] Because the vein grafts were in a high flow situation, we did not measure graft blood flow or peripheral vascular resistance. We therefore cannot comment on how the possible effect of levcromakalim to reduce peripheral vascular resistance might have influenced our results; this property of levcromakalim is likely to assume greater importance for the properties of femoral artery vein grafts. Neither are we able to comment on any morpho-

Table II. Morphometric measurements and cell count in vein grafts at 4 weeks

	Placebo (n = 7)	*Levcromakalim (n = 8)*	P*
Intimal area (mm^2)	0.33 ± 0.17	0.06 ± 0.02	.001
Medial area (mm^2)	0.59 ± 0.11	0.60 ± 0.19	.949
Intimal:medial area ratio	0.60 ± 0.33	0.10 ± 0.02	.001
Lumen area (mm^2)	1.88 ± 0.51	2.32 ± 0.46	.04
Vein graft size (mm^2)	2.81 ± 0.69	2.97 ± 0.45	.59
Intimal cell density (cells/mm^2)	3230 ± 580	2300 ± 350	.001
Medial cell density (cells/mm^2)	3020 ± 570	2860 ± 340	.52

**P* calculated by Mann-Whitney.

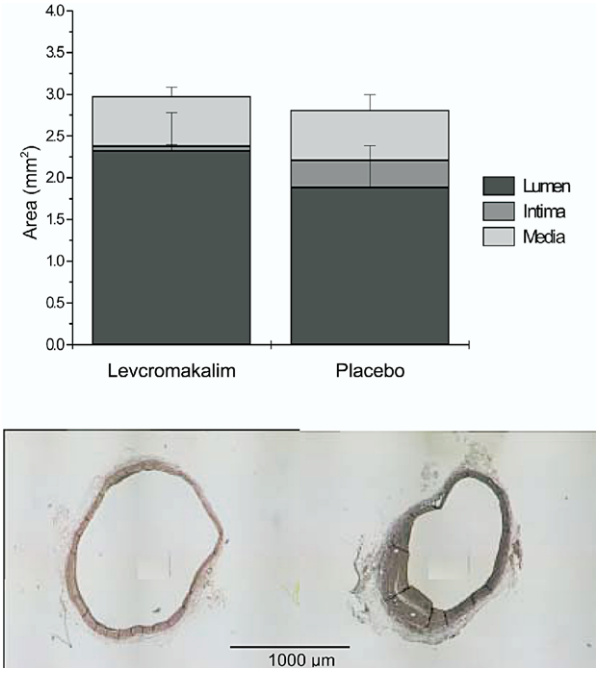

Fig 7. Summary of the morphometric data at 4 weeks and characteristic cross-section of vein grafts from the levcromakalim and placebo groups. Example cross-sections of grafts are shown below the summary data with levcromakalim on the left and placebo on the right. Data are means ± SD.

logic changes observed in the adventitia, because laboratory processing caused adventitial detachment in a few samples. Because our study was directed at identifying a pharmacologic alternative to external stenting, it is relevant that external stenting also causes expansive remodelling in an atherosclerotic model using apoE(-/-) mice.[30]

The possibility remains that the early expansive remodelling of vein grafts would be associated with aneurysmal dilatation with longer follow-up, later atherosclerotic changes in the vein graft, or further important hemodynamic changes. The long-term follow up that would be necessary to investigate these matters in experimental vein grafts is not condoned by our regulatory authorities. For now, we only can rely on the good evidence to indicate that the remodelling of experimental vein grafts is complete by 4 weeks.[15]

There are several possibilities for further studies to explore in more detail the molecular mechanisms whereby levcromakalim causes expansive remodelling of vein grafts. These include more detailed immunochemical analysis of the 1-week vein graft sections for relevant markers, such as caspase[3,21] and quantification of macrophages and other types of inflammatory cells. If translation to patients is considered, the next steps should include a trial of levcromakalim in an atherosclerotic model using end-to-side anastomoses.[30]

CONCLUSION

We have identified a simple treatment associated with the expansive remodeling of experimental vein grafts. This goal of expansive remodelling is likely to have greater clinical relevance than the goal of reduction of intimal hyperplasia.

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AUTHOR CONTRIBUTIONS

Conception and design: MG, GWT, JTP
Analysis and interpretation: LW, GWT, MG, JTP
Data collection: LW
Writing the article: LW, JTP
Critical revision of the article: AHD, GWT
Final approval of the article: All authors
Statistical analysis: Acknowledged
Obtained funding: Acknowledged
Overall responsibility: LW

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